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Ceftriaxone, a beta-lactam antibiotic, attenuates relapse-like ethanol-drinking behavior in alcohol-preferring rats

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Abstract

Relapse-like ethanol-drinking behavior depends on increased glutamate transmission in the mesocorticolimbic motive circuit. Extracellular glutamate is regulated by a number of glutamate transporters. Of these transporters, glutamate transporter 1 (GLT1) is responsible for the majority of extracellular glutamate uptake. We have recently reported that ceftriaxone (CEF) treatment (i.p.), a β -lactam antibiotic known to elevate GLT1 expression, reduced ethanol intake in male alcohol-preferring (P) rats. We investigated here whether CEF treatment attenuates relapse-like ethanol-drinking behavior. P rats were exposed to free choice of 15% and 30% ethanol for 5 weeks and treated with CEF (50 and 100 mg/kg, i.p.) during the last 5 days of the 2-week deprivation period. Rats treated with CEF during the deprivation period showed a reduction in ethanol intake compared with saline-treated rats upon re-exposure to ethanol; this effect persisted for 9 days. Moreover, CEF-mediated attenuation in relapse to ethanol-drinking behavior was associated with upregulation of GLT1 level in prefrontal cortex and nucleus accumbens core. GLT1 upregulation was revealed only at the higher dose of CEF. In addition, CEF has no effect on relapse-like sucrose-drinking behavior. These findings suggest that ceftriaxone might be used as a potential therapeutic treatment for the attenuation of relapse-like ethanol-drinking behavior.

Keywords

Relapse; glutamate; ethanol intake; EAAT2; alcohol dependence

Introduction

Relapse to ethanol use after prolonged withdrawal periods represents a significant problem in the treatment of alcohol addiction. Evidence suggests that activation of the mesocorticolimbic system constitutes a common neurocircuitry of drug intake by which drugs of abuse can mediate acute reinforcing effects. Long-term neuroadaptations in this circuitry may underlie the transition to drug dependence and cycles of relapse (Feltenstein and See, 2008). Neuroadaptive changes in the glutamatergic system can lead to hyperexcitability of the central nervous system during the withdrawal period, which suggested that it is one of the mechanisms responsible for alcohol craving (for review, see

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Conflict of interest

The authors declare that there are no conflict of interest.

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Gass and Olive, 2008). Glutamate transmission and its neuroadaptive changes are critical in relapse-like seeking behavior (for review, see Tzschentke and Schmidt, 2003). It is noteworthy that two brain regions of the mesocorticolimbic system, the prefrontal cortex (PFC) and nucleus accumbens (NAc), are key players in relapse to drug abuse (Capriles et al., 2003; Childress et al., 1999; Goldstein and Volkow, 2002; McFarland et al., 2003; Shalev et al., 2002). This involves the release of glutamate from the PFC projection to the NAc. Studies have demonstrated that the levels of extracellular glutamate are increased in NAc during ethanol consumption (Melendez et al., 2005; Selim and Bradberry, 1996; Szumlinski et al., 2007). In addition, chronic ethanol consumption induced a decrease in the function of glutamate transport in the cerebral cortex (Schreiber and Freund, 2000). These studies suggest that chronic ethanol exposure leads to an increase in glutamate output and impairs ability in glutamate transport, leading ultimately to excessive glutamatergic neurotransmission within the mesocorticolimbic circuit.

Brain extracellular levels of glutamate are regulated by glutamate transporters, which are found in neuronal and glial membranes, and they regulate extracellular glutamate levels below the excitotoxic levels (Rothstein et al., 1996). Among these transporters, glutamate transporter 1 (GLT1; its human homologue excitatory amino acid transporter 2, EAAT2) is responsible for the removal of the majority of extracellular glutamate (Mitani and Tanaka, 2003; Rothstein et al., 1995). Importantly, we recently reported that treatment with ceftriaxone (CEF), a β -lactam antibiotic known to elevate GLT1 expression, decreased ethanol intake in male alcohol-preferring (P) rats (Sari et al., 2011). The reduction of ethanol intake was associated, in part, with upregulation of GLT1 in PFC and NAc. Since relapse-like ethanol-drinking behavior depends on increased glutamate transmission in key regions of the mesocorticolimbic circuit, we tested whether CEF-induced GLT1 upregulation would attenuate relapse-like ethanol-drinking behavior in P rats. P rats develop physical dependence when ethanol is withdrawn, and they show an alcohol deprivation effect (ADE) that can be extended after several weeks of ethanol deprivation (Rodd-Henricks et al., 2000, 2001; Sari et al., 2006). In this study, P rats had access to free choice of 15% and 30% ethanol for 5 weeks, and then they were deprived for 2 weeks. During the last 5 days of the 2-week deprivation, rats were treated with CEF (50 or 100 mg/kg, i.p.) or saline. Rats were then re-exposed to ethanol for 9 days during the relapse paradigm. Daily body weight, water, and ethanol consumption were measured during the re-exposure paradigm. We further investigated whether CEF-mediated attenuation in relapse-like ethanol-drinking behavior was associated in part with upregulation of GLT1 level in the PFC and NAc core. We focused on the NAc core instead of the NAc shell based on findings demonstrating that cocaine-induced release of glutamate was more pronounced in the NAc core than in the shell (Pierce et al., 1996). Moreover, the NAc core was found to be associated with reinstatement of cocaine-seeking behavior (McFarland et al., 2004). We also have tested whether CEF treatment has any effect on relapse-like sucrose-drinking behavior as a control of appetitive-motivated behavior.

Materials and methods

Animals

Data reported in this study were obtained from adult male P rats. P rats have been well characterized both behaviorally and neuro-biologically (Li et al., 1979; McBride and Li, 1998), and satisfy criteria proposed as essential for an animal model of alcohol dependence (Lester and Freed, 1973). P rats can consume pharmacologically relevant levels of ethanol (McBride and Li, 1998; Murphy et al., 2002), which makes them ideal animal models for testing and identifying therapeutic targets for treatment of relapse-like ethanol-drinking behavior. P rats were obtained from the Indiana University Medical Center at approximately 4 weeks of age. Animals started with the experimental procedures at approximately 90 days;

their body weight averaged 317–395 g. Three experimental groups were divided randomly and studied for relapse-like ethanol-drinking behavior: (1) a saline control group ($n = 8$); (2) CEF 50 mg/kg-treated group ($n = 8$); and (3) CEF 100 mg/kg-treated group ($n = 8$). These doses of CEF were tested based on recent studies that investigated the effects of CEF on ethanol intake in P rats (Sari et al., 2011). CEF was dissolved in saline; a total of 0.5 mL/0.5 kg of body weight was injected into each animal. In addition, three separate experimental groups were divided randomly and examined for sucrose-drinking behavior: (1) saline control group ($n = 4$); (2) CEF 50 mg/kg-treated group ($n = 5$); and (3) CEF 100 mg/kg-treated group ($n = 5$). After habituation to the vivarium, P rats were individually housed in bedded plastic cages in a temperature- (21°C) and humidity-controlled (~50%) vivarium that was maintained on a 12/12-hour light (0700–1900)/dark (1900–0700) cycle. All animals had ad lib access to food and water. Note that all animal procedures were approved by the Institutional Animal Care and Use committee of The University of Toledo (animal protocol #106966) in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996). The Institution is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALACI) and is in compliance with municipal, state and federal laws and regulations governing animal scientific research.

Behavioral drinking paradigms

At approximately 3 months old, male P rats were given continuous free choice access to food, water and two bottles that offered a choice of ethanol at different concentrations (15% and 30% v/v) for 5 weeks. For 5 weeks of continuous ethanol access, ethanol consumption for each animal was measured as grams of ethanol consumed per kilogram of body weight per day. Ethanol measurements were made by subtracting the weight of the bottle from its previous weight. At the end of the 5 weeks, animals that did not meet the requirement of drinking more than or equal to 4 g/kg/body weight averaged across the last 2 weeks of continuous ethanol exposure were removed from the study, based on criteria we adopted in a recent study (Sari et al., 2011). Baseline calculations were the average measurements taken across the last 2 weeks of the 5-week ethanol and water intakes along with body weights. Ethanol and water intake and body weights were measured three times per week during the 5-week continuous ethanol-drinking paradigm. On week 6, animals were designated randomly into three different groups and deprived of ethanol only for 2 weeks. During the last 5 days of the 2-week deprivation period, animals received CEF 50 mg/kg (i.p.), CEF 100 mg/kg (i.p.) or saline once a day for five consecutive days. After the 5 days of treatment, animals were re-exposed to free choice 15% and 30% ethanol for 9 days. During this period of re-exposure to ethanol, body weights of animals and water and ethanol intake were recorded daily. P rats were euthanized on day 10, and brains were removed and frozen at -70°C .

Sucrose-drinking paradigms

We further tested the effects of CEF on relapse-like sucrose-drinking behavior in separate groups of male P rats. Three sucrose groups were divided randomly and had continuous access to 10% sucrose for 3 weeks. Sucrose intake, water consumption and animal body weights were measured three times per week during the 3-week continuous sucrose-drinking paradigm. On week four, animals were deprived of sucrose for 2 weeks. During the last 5 days of the 2-week deprivation period, each group of animals received CEF 50 mg/kg (i.p.), CEF 100 mg/kg (i.p.) or saline (i.p.) once a day for five consecutive days. After 5 days of treatment, animals were re-exposed to sucrose-drinking paradigm for 9 days to determine the specificity of CEF treatment in relapse-like ethanol-drinking behavior. During this

period, daily measurements of animal body weights, and water and sucrose intake were recorded.

Brain tissue harvesting

On the last day of the relapse-like ethanol-drinking paradigm, animals were euthanized by isoflurane, rapidly decapitated with a guillotine, and their brains were dissected and immediately frozen on dry ice and stored at -70°C .

As described in detail in a recent study from our lab, the PFC and the NAc core were extracted after they were micropunched stereotactically using a cryostat apparatus that was maintained at -20°C , which kept the tissue frozen (Sari and Sreemantula, 2012). We used the rat brain stereotaxic coordinates to identify and dissect the PFC and NAc core (Paxinos and Watson, 1986). The NAc was identified with the appearance of anterior commissure. We used surgical blades to isolate the NAc core and the PFC with the visualization of landmarks. The medial part of the PFC was selected and dissected at the same level of the NAc core. Both these brain regions were extracted according to the Paxinos and Watson atlas of the rat brain. These regions were then frozen at -70°C for Western blot assay to detect GLT1 and β -tubulin levels.

Western blot for GLT1 expression

GLT1 levels were determined in PFC and NAc core in saline vehicle and CEF-treated groups from randomly selected groups using Western blot assay as recently described (Sari et al., 2009, 2010, 2011). In brief, brain regions (PFC and NAc core) were homogenized in filtered lysis buffer, and protein was extracted and quantified for Western blot assay. Extracted proteins were prepared with 5 $\times$ Laemmli loading dye and loaded in a 10–20% glycine gel (Invitrogen). Separated proteins in the gels were transferred electrophoretically onto a nitrocellulose membrane at 30 V for 1 h. Membranes were blocked using 3% milk in Tris-buffered saline Tween-20 for 30 min at room temperature. The membranes were then incubated with guinea pig anti-GLT1 antibody (Millipore Bioscience Research Reagents, Inc.) at a 1:5000 dilution in blocking buffer at 4°C overnight and later incubated with horseradish peroxidase (HRP)-labeled anti-guinea pig secondary antibody (1:5000 dilution). We used β -tubulin antibody as a loading control protein. SuperSignal West Pico kit (Pierce, Inc.) was used for chemiluminescent detection of HRP, and the membrane was then exposed to Kodak BioMax MR film (Thermo Fisher Scientific). Films were developed on an SRX-101A machine, and digitized images of immunoblots were quantified with the MCID system. Data are reported as percentage ratios of GLT1/ β -tubulin, relative to saline control (100% control-value) levels.

Statistical analyses

Two-way mixed analysis of variance (ANOVA) was performed for statistical analysis of ethanol, water, and sucrose intake and animal body weight data. When a significant main effect of day and interaction effect (Day by Treatment) were observed, we conducted one-way ANOVA analyses between saline vehicle control and CEF-treated groups using post-hoc Dunnett's (two-sided) test. Immunoblots data were statistically analyzed using one-way ANOVA and Newman-Keuls's test for comparison between saline vehicle control and CEF-treated groups. All statistical tests were based on $p < 0.05$ level of significance.

Results

Ceftriaxone treatment on ethanol intake during relapse-like ethanol-drinking behavior

During the 9 days of re-exposure to ethanol after 5 days of treatment with saline, CEF 50 mg/kg or CEF 100 mg/kg, we measured daily ethanol consumption (g/kg/day). Figure 1(A)

shows the average daily ethanol intake during 9 days of ethanol re-exposure for groups treated with saline, CEF 50 mg/kg, and CEF 100 mg/kg. Note that the baseline was calculated by averaging the amount of ethanol intake across the last 2 weeks of the 5-week ethanol-drinking period prior to re-exposure to ethanol for relapse-like behavior. A 3×9 (Dose by Day) two-way mixed ANOVA analysis conducted on ethanol intake, followed by a Dunnett post-hoc test, revealed a significant Dose by Day interaction [$F(2,18) = 5.38, p < 0.001$] and a significant main effect of day [$F(1,9) = 23.88, p < 0.001$] and Dose (treatment) [$F(2,21) = 46.56, p < 0.01$]. Simple effect analyses, conducted as one-way ANOVAs for each day, revealed significant [$F > 15.55, p < 0.001$] differences among the doses (treatments) for days 1 through 9. Dunnett's post-hoc test revealed that both CEF doses significantly decreased ethanol intake on days 1 through 9 of re-exposure as compared with saline. Alternatively, Dunnett's post-hoc analyses demonstrated that the amount of ethanol intake on Day 1 is significantly different as compared with the amount of ethanol intake corresponding to the baseline in the saline group ($p < 0.05$). There were no significant differences in ethanol intake between baseline and the other days (2–9). There also were no significant differences in the baselines among all groups.

Ceftriaxone treatment on water intake during relapse-like ethanol-drinking behavior

We have also determined the effect of CEF on water intake during relapse-like ethanol-drinking paradigm from days 1–9. Note that the baseline was calculated by averaging the amount of water intake across the last 2 weeks of the 5-week ethanol-drinking period prior to re-exposure to ethanol for relapse-like behavior. A 3×9 (Dose by Day) two-way mixed ANOVA conducted on daily water intake (Figure 1(B)), followed by a Dunnett post-hoc test, revealed a significant Dose by Day interaction [$F(2,18) = 2.32, p < 0.003$] and a significant main effect of day [$F(1,9) = 6.45, p < 0.001$] and Dose (treatment) [$F(2,21) = 21.91, p < 0.01$]. Simple effect analyses, conducted as one-way ANOVAs for each day, revealed significant [$F > 3.65, p < 0.05$] differences among the doses (treatments) for days 2 through 9. Dunnett's post-hoc test revealed that both CEF doses, relative to saline, significantly increased water intake on days 2 through 9. There were no significant differences in water intake between baseline and days of re-exposure to ethanol. There also were no significant differences in the baselines among all groups.

Ceftriaxone treatment on body weight during relapse-like ethanol-drinking behavior

We also have determined the effect of CEF on the body weight of all animals. A 3×9 (Dose by Day) two-way mixed ANOVA conducted on body weight (Figure 2(A)), followed by a Dunnett post-hoc test, revealed a significant Dose by Day interaction [$F(2,18) = 3.369, p < 0.001$] and a significant main effect of day [$F(1,9) = 68.251, p < 0.001$]. However, there was no significant main effect of Dose (treatment) [$F(2,21) = 0.27, p = 0.76$]. Moreover, simple effect analyses, conducted as one-way ANOVAs for each day, revealed no significant differences [$F < 0.725, p > 0.496$] among the doses (treatments) from days 1 through 9. There were no significant differences in body weight between the baseline and days of re-exposure to ethanol. These results indicate that CEF did not affect body weight.

Ceftriaxone treatment on sucrose consumption during relapse-like sucrose-drinking behavior

We further tested the effect of CEF on sucrose intake as an appetitive control for motivation to behavioral drinking (Figure 2(B)). A 3×9 (Dose $\times$ Day) two-way mixed ANOVA, followed by a Dunnett post-hoc test, revealed a non-significant Dose by Day interaction [$F(2,18) = 0.173, p = 1.00$] and a significant main effect of day [$F(1,9) = 4.152, p < 0.001$]. In addition, there was no significant main effect of Dose (treatment) [$F(2,11) = 0.44, p = 0.65$]. Simple effect analyses, conducted as one-way ANOVAs for each day, demonstrated no significant [$F < 0.514, p > 0.612$] differences among the doses (treatments). These data

indicate that CEF did not affect sucrose intake. Note that there were no significant differences in sucrose intake between the baselines and days of re-exposure to sucrose. In addition, there were no significant differences in the baselines among all groups. It is noteworthy that there was no variability in sucrose drinking among all groups, as they were drinking most of the solution provided to them.

Ceftriaxone treatment on GLT1 expression in PFC and NAc core

We next examined the effects of CEF treatment on GLT1 levels in PFC using Western blot (Figure 3, upper panel). We have used β -tubulin as a control loading protein. Statistical analysis using one-way ANOVA demonstrated a significant main effect between control and CEF treatment groups [$F(3, 14) = 3.88, p < 0.05$]. A post-hoc test, Newman–Keuls, revealed a significant increase in the percent ratio of GLT1/ β -tubulin in the CEF 100 mg/kg-treated group compared with the saline-treated group ($p < 0.05$, Figure 3, lower panel). There was no significant difference between CEF 50 mg/kg and CEF 100 mg/kg-treated groups or between saline and CEF 50 mg/kg-treated groups.

We also have examined the effects of CEF treatment on GLT1 levels in NAc core using Western blot (Figure 4, upper panel). We have used β -tubulin as a control loading protein. Statistical analysis using one-way ANOVA demonstrated a significant main effect between saline control and CEF treatment groups [$F(3, 14) = 8.03, p < 0.01$]. A post-hoc test, Newman–Keuls, showed a significant increase in the % ratio of GLT1/ β -tubulin in the CEF-100 mg/kg compared with CEF-50 mg/kg ($p < 0.05$) and saline control ($p < 0.01$) groups (Figure 4, lower panel). There was no significant difference between saline and CEF-50 mg/kg-treated groups.

Discussion

We report here that treatment with CEF 50 mg/kg and CEF 100 mg/kg significantly attenuates relapse-like ethanol-drinking behavior compared with the saline-treated group. The reduction in ethanol consumption during re-exposure to ethanol (relapse-like ethanol-drinking behavior) was correlated with upregulation of GLT1 level in the NAc core and PFC brain regions at the higher dose of CEF. However, the findings with the lower dose of CEF support the hypothesis that CEF may have other pharmacological actions. Alternatively, CEF treatment did not affect sucrose intake, indicating CEF's specificity in attenuating relapse-like ethanol-drinking behavior. Furthermore, there were significant differences in water intake between saline and CEF-treated groups; however, there was no significant difference in the body weight among all the tested groups, indicating that CEF did not affect body weight of animals. The increase in water intake for CEF-treated groups might compensate for the decrease in their ethanol intake during relapse-like behavior.

We tested the effects of CEF in relapse-like behavior using the P rat, which is an animal model that has been shown to develop physical dependence after ethanol withdrawal (Kampov-Polevoy et al., 2000; Waller et al., 1982). It is noteworthy that P rats cannot show an ADE when deprivation to ethanol occurs (Rodd-Henricks et al., 2000; Sari et al., 2006). ADE is considered a temporary increase in ethanol intake after periods of deprivation (for review, see Le and Shaham, 2002). The ADE was found to be dependent on cycles of deprivation and the duration of the deprivation period (Heyser et al., 1997; Sinclair et al., 1973; Spanagel and Holter, 2000). It is noteworthy that studies have shown that ADE was associated with increased ethanol intake in Wistar and P rats, but no ADE was observed in high-alcohol-drinking (HAD) and ALKO-alcohol (AA) rats after deprivation (Vengeliene et al., 2003). However, these rats (HAD and AA) showed increases in their preference for consuming higher concentrations of ethanol. In regards to P rats used in this study, studies confirmed that these P rats show all the phenotypes for relapse-like ethanol-drinking

behavior (Bell et al., 2006; Murphy et al., 2002). The ADE has been used to assess relapse-like behavior in P rats (Rodd-Henricks et al., 2000, 2001). After a single deprivation period of 2–8 adult P rats increased ethanol intake from 5 g/kg body weight/day in 24-h free choice access to 10% ethanol, just prior to deprivation, to around 9 g/kg body weight/day on the first day of re-exposure to ethanol (Rodd-Henricks et al., 2000). Thus, we used P rats in this study to determine the effects of CEF-induced upregulation of GLT1 in relapse-like ethanol-drinking behavior.

The glutamatergic system has long been suggested to play a key role in drug addiction and alcoholism, and this includes relapse-like behavior (for review, see Gass and Olive, 2008; Vengeliene et al., 2008). The importance of the glutamatergic system in relapse-like ethanol-drinking behavior was revealed by several studies, including one that tested acamprosate, a drug that reduces glutamatergic activity, and has shown reduction in the ADE in Wistar rats (Heyser et al., 1998; Holter et al., 1997; Spanagel et al., 1996). Moreover, studies have demonstrated that treatment with competitive and non-competitive N-methyl-D-aspartate (NMDA) receptor antagonists attenuated relapse-like ethanol-drinking behavior (Holter et al., 2000; Vengeliene et al., 2005). Furthermore, treatment with metabotropic glutamate receptor 5 (mGluR5) antagonist reduced relapse-like ethanol self-administration in Wistar rats and reduced repeated ADEs in P rats (Backstrom et al., 2004; Schroeder et al., 2005). These findings suggest the important role of the glutamatergic system in relapse-like ethanol-drinking behavior.

It is noteworthy that local striatal stimulation of the NMDA receptor increased extracellular glutamate levels in rats withdrawn from chronic ethanol exposure (Rossetti et al., 1999). These results suggest that glutamate-induced NMDA receptor-mediated elevations of extracellular glutamate during ethanol withdrawal may contribute to the neuropathology associated with alcoholism. Moreover, studies demonstrated that a blockade of AMPA/kainate receptors in NAc attenuates relapse-like drug-seeking behavior (Cornish and Kalivas, 2000; Park et al., 2002). Alternatively, studies revealed that chronic ethanol consumption led to down-regulation of glutamate transporter function in the cerebral cortex of alcohol-preferring cAA rats (Schreiber and Freund, 2000). It is noteworthy that dysregulation of glutamate transmission in PFC–NAc pathways is a key factor in uncontrolled seeking behavior to drug abuse (for review, see Kalivas et al., 2005). We used CEF, a β -lactam antibiotic, to induce upregulation of GLT1 because CEF appears to have a direct central effect on glutamate transporter function (Rothstein et al., 2005; Sari et al., 2011). Previous studies by Rothstein et al. (2005) reported that rats treated at a dose of 200 mg/kg (i.p) daily for 5 days increased levels of GLT1 and functional activity in both the spinal cord and the hippocampus. Furthermore, our laboratory has reported that CEF attenuates cue-induced relapse to cocaine-seeking behavior in a dose-dependent manner (Sari et al., 2009). In accordance, it was also demonstrated that CEF treatment indeed reduced a cue-induced relapse to cocaine (Knackstedt et al., 2010). This attenuation of cue-induced relapse to cocaine-seeking behavior was associated with upregulation of GLT1 levels in PFC and NAc.

Since GLT1 regulates the uptake of the majority of extracellular glutamate, we suggest here that an increase in this uptake might be modulated through upregulation of GLT1 that would attenuate relapse-like ethanol-drinking behavior. Studies from our lab and others demonstrated that CEF administration for 5 days can lead to upregulation of GLT1 levels (Rothstein et al., 2005; Sari et al., 2009, 2011). In this study, we report a correlation, with the higher dose of CEF, between increased GLT1 protein expression in the NAc core and PFC and the attenuation in relapse-like ethanol-drinking behavior. It is important to note that the roles of PFC and NAc during craving for drugs of abuse, including ethanol and cocaine, have been revealed by neuroimaging studies (Dom et al., 2005; Garavan et al., 2000;

Goldstein and Volkow, 2002; Wexler et al., 2001). We investigated the effect of CEF on GLT1 upregulation in the NAc core instead of the NAc shell based on previous findings that cocaine-induced release of glutamate was more pronounced in the NAc core than in the shell of rats developing behavioral sensitization (Pierce et al., 1996). In addition, increased release of glutamate from the PFC–NAc projections, in particular the NAc core, was associated with reinstatement of cocaine-seeking behavior (McFarland et al., 2004). The NAc core is a brain region that is involved in drug abuse, including ethanol; this brain region involves learning drug-seeking behaviors (for review, see Kalivas et al., 2005; Kalivas and Volkow, 2005).

Moreover, in this study, CEF 50 mg/kg did not upregulate GLT1 level, but it did attenuate relapse-like ethanol-drinking behavior. This effect was also observed in a recent study that demonstrated the effect of lower doses of CEF on the reduction of ethanol intake, which was not correlated with upregulation of GLT1 in central reward brain regions, at least with the Western blot method used in this study and a recent study (Sari et al., 2011). However, in this recent study, we demonstrated that higher doses of CEF (100 and 200 mg/kg) have been found to induce a long-lasting effect on the attenuation of ethanol intake as compared with the lower dose of CEF (50 mg/kg). This suggests the importance of GLT1 upregulation in the long-lasting effect of reduction of ethanol intake. It is unclear whether CEF50 has an effect on glutamate uptake without observing any GLT1 upregulation. Thus, studies are warranted to determine the effect of CEF50 on glutamate uptake. It is noteworthy that, in addition to CEF's effects on GLT1, this drug may have other pharmacological effects on the attenuation of ethanol drinking or relapse-like ethanol-drinking behavior. Other pharmacological effects may include oxidative stress components. For example, chronic ethanol exposure was shown to be linked with a decrease in brain glutathione (GSH) and an increase in oxidized glutathione in vivo (Gotz et al., 2001). Furthermore, ethanol withdrawal was found to be associated with increases in oxygen-derived free radicals which, by oxidation of thiol groups located on the glutamate transporters, may block the glutamate uptake (Volterra et al., 1994). Importantly, an in vitro study demonstrated that CEF increased both GSH and cysteine/glutamate exchanger (xCT) levels, leading to the reversal of the glutamate transporter deficits caused by the increased levels of free radical oxidation (Lewerenz et al., 2009). In addition, an in vivo rat model study demonstrated that CEF treatment increased the levels of xCT and GLT1 in NAc (Knackstedt et al., 2010). Together, these findings suggest that in addition to CEF-induced upregulation of GLT1, the drug may have an upregulatory effect on xCT level and resulting in a subsequent increase in the levels of GSH, which can lead to the attenuation of relapse-like ethanol-drinking behavior. Studies are warranted to determine the levels of xCT and GSH in this relapse-like ethanol-drinking behavior model.

We conclude here that CEF attenuates relapse-like ethanol-drinking behavior in male P rats. We suggest here, based on findings with the higher dose of CEF, that upregulation of GLT1 may overcome impaired basal extracellular glutamate levels in central reward brain regions, including NAc (Knackstedt et al., 2010). Although CEF may act through unknown mechanisms of action, the results with the higher dose of CEF suggest that GLT1 may be a potential target for attenuating relapse-like ethanol-drinking behavior.

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Figure 1.

A) Daily ethanol intake of male P rats for nine days of ethanol re-exposure, following five days of treatment during relapse period with saline ($n = 8$), CEF 50 mg/kg ($n = 8$) and CEF 100 mg/kg ($n = 8$). The graph represents average daily ethanol intake during the 9 days of ethanol re-exposure. The baseline values correspond to the average ethanol intake across the last 2 weeks of 5-week ethanol exposure. A one-way ANOVA, followed by a Dunnett post-hoc test, revealed a significant reduction in average daily ethanol consumption during the duration of re-exposure to ethanol for both CEF-treated groups as compared with the saline-treated group. B) Daily water intake of male P rats for 9 days of relapse paradigm, following 5 days of treatment with saline ($n = 8$), CEF 50 mg/kg ($n = 8$), or CEF 100 mg/kg ($n = 8$). The graph represents average daily water consumption during the 9 days of ethanol re-exposure. The baseline values correspond to the average water intake across the last 2 weeks

of the 5-week ethanol exposure. A one-way ANOVA, followed by a Dunnett post-hoc test, revealed a significant increase in average daily water consumption during the duration of re-exposure to ethanol for both CEF-treated groups as compared to the saline-treated group. Values shown as means $\pm$ SEM. * $p < 0.001$ (A) and * $p < 0.05$ (B) depict a significant difference across doses for each day.

Figure 2.

A) Daily body weight measurement of male P rats for 9 days, following 5 days of treatment with saline ($n = 8$), CEF 50 mg/kg ($n = 8$), or CEF 100 mg/kg ($n = 8$). The graph represents average daily body weight during the 9 days of ethanol re-exposure. A one-way ANOVA, followed by a Dunnett post-hoc test, demonstrated no significant effect of dose and day during the duration of re-exposure for all groups. B) Daily sucrose (10%) intake of male P rats for 9 days, following 5 days of treatment with saline ($n = 4$), CEF 50 mg/kg ($n = 5$), or CEF 100 mg/kg ($n = 5$). The graph represents average daily sucrose (10%) consumption ($\pm$ SEM) during the 9 days of sucrose (10%) re-exposure. While the Day main effect was significant ($p < 0.001$), neither the interaction by Day and Dose (treatment) ($p = 1.00$) nor the Dose main effect ($p > 0.612$) was significant. In addition, there were no significant

differences in sucrose intake between the baseline and days of re-exposure to sucrose. Values shown as means $\pm$ SEM.

Figure 3.

Effects of 50 mg/kg (CEF-50, $n = 4$), 100 mg/kg (CEF-100, $n = 4$) or saline treatment ($n = 4$) on GLT1 expression in the PFC. (Upper panel) Immunoblots for GLT1 expression and β -tubulin as a control loading protein. (Lower panel) Quantitative analysis of immunoblots demonstrated a significant increase in the % ratio GLT1/ β -tubulin in CEF-100-treated groups compared with saline control (100% control-value) group. There was neither a significant difference between CEF-100 and CEF-50 groups nor between saline vehicle and CEF-50 groups. Values shown as means $\pm$ SEM. (* $p < 0.05$).

Figure 4.

Effects of 50 mg/kg (CEF-50, $n = 4$), 100 mg/kg (CEF-100, $n = 4$) or saline treatment ($n = 4$) on GLT1 expression in the NAc core. (Upper panel) Immunoblots for GLT1 expression, and β -tubulin as a control loading protein. (Lower panel) Quantitative analysis of immunoblots demonstrated a significant increase in the ratio of GLT1/ β -tubulin in CEF-100 treated group as compared with CEF-50 and saline control (100% control-value) groups. There was no significant difference between saline vehicle and CEF-50 groups. Values shown as means $\pm$ SEM. (* $p < 0.05$; ** $p < 0.01$).